Mutations of the RAG3 gene encoding a regulator of fermentation in Kluyveromyces lactis are suppressed by a mutation of the transcription factor gene KLGCR1

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Received 28 November 2006, revised 4 January 2007, accepted 4 January 2007.
First published online 9 June 2007.

DOI:10.1111/j.1567-1364.2007.00219.x

Editor: Hiroshi Fukuhara

Keywords
pyruvate decarboxylase; transcriptional regulation; genetic suppressor.

Abstract
In Kluyveromyces lactis, Rag3 regulates both fermentative metabolism and thiamine biosynthesis. Regulation of fermentation is exerted at the level of transcription of KIPDC1. We have isolated and identified a mutation of the transcription factor KlGCR1, Klgcr1-1, which suppressed the fermentative-deficient phenotype associated with the RAG3 deletion. In the mutant, the transcription of KIPDC1 was restored. However, we found that the suppression was not specific to the RAG3 mutation, as the Klgcr1-1 mutation could also suppress the fermentative defect associated with mutation of Sck1, another regulator of glycolysis.

Introduction

Kluyveromyces lactis is an aerobic respiratory yeast in which fermentation is facultative and that displays no redundancy in most of the genes involved in glucose metabolism. Nevertheless, in the presence of high extracellular glucose concentrations, the cells can ferment and are able to grow even if respiration is blocked by antimycin A (Rag+ phenotype) (Goffrini et al., 1989). This results from the enhancement of the rate-limiting step of glucose metabolism, its transport into the cells, by inducing the expression of the RAG1 gene encoding a low-affinity glucose permease (Węsolowski-Louvel et al., 1992a).

In K. lactis, a single gene, KIPDC1 (RAG6), codes for pyruvate decarboxylase (PDC) (Bianchi et al., 1996). The consequences of KIPDC1 mutation are: (1) absence of PDC activity and ethanol production; and (2) the Rag+ phenotype, i.e. absence of fermentative growth. However, growth on glucose is barely affected when respiration is not impeded. The expression of KIPDC1 is regulated at the level of transcription by several factors, including carbon source (glucose induction), oxygen availability, glycolytic regulatory genes (KIGCR1, KIGCR2, SCK1, RAG8), and autoregulation by its own product (Bianchi et al., 1996; Destruelle et al., 1999; Lemaire et al., 2002; Nei et al., 2004).

Transcription of KIPDC1, and consequently PDC activity and ethanol production, are also dependent on RAG3 (Prior et al., 1996), a homolog of PDC2 of Saccharomyces cerevisiae (Hohmann, 1993). Rag3, like Pdc2, is required for the expression of THI genes involved in the biosynthesis of thiamine (Hohmann & Meacock, 1998; Tizzani et al., 1998; Mojzita & Hohmann, 2006), the cofactor of PDC and several key enzymes. However, little is known about the mechanism by which Rag3 and Pdc2 regulate their target genes. The presence in both proteins of a sequence similar to that of centromere-binding protein, CENP-B, suggests that they would bind to DNA.

In this study, using a genetic approach, we tried to obtain an insight into the mechanism of KIPDC1 regulation by Rag3. We looked for genetic suppressors of the rag3 mutation. This allowed us to identify recessive mutations in the...
KIGCR1 gene, coding for a regulator of glycolytic gene expression, that bypass the need for Rag3 for growth on high-glucose media in the presence of antimycin A. One of these mutations, Klgcr1-1, results in a truncated KIGcr1-1 protein that is still able to bind glycolytic gene promoters in vitro and that does not affect KIGcr1-dependent transcription of glycolytic genes.

## Materials and methods

### Strains and plasmids

The yeast strains used in this work are listed in Table 1. Disruption of RAG3 (strain MWK23, Table 1) was done by integrative transformation with a HindIII fragment of plasmid pT218 containing the S. cerevisiae LEU2 gene inserted into the Swal/EcoRV sites of a 1.93-kb HindIII fragment of the RAG3 locus (Prior et al., 1996).

The plasmids used in this work are listed in Table 2. The pGEMgcrl-1 plasmid was constructed by cloning the PCR-amplified Klgr1-1 allele into the pGEM–T Easy vector (Promega). The oligonucleotides used for the amplification were: 5’-CCACGTAGGGGAAAATTT-3’ and 5’-TACCACTGATTCTGTTTTCA-3’. Two clones from independent amplification reactions were sequenced on both strands. Plasmid pHN14-1 was constructed by cloning the 2-kb SalI–PstI fragment from plasmid pGEMgcrl-1, containing most of the 3’-coding region of the mutant allele Klgr1-1, into pMAL-cRI (New England Biolabs). The construction resulted in an in-frame fusion of malE with the 672 last codons of Klgr1-1.

### Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM6-7A</td>
<td>MATa adeT-600 uraA1-1</td>
<td>Wesolowski-Louvel et al. (1992b)</td>
</tr>
<tr>
<td>PM6-7AVV23</td>
<td>MATa adeT-600 uraA1-1 rag3-1</td>
<td>Wesolowski-Louvel et al. (1992b)</td>
</tr>
<tr>
<td>PM6-7AVV43</td>
<td>MATa adeT-600 uraA1-1 rag3-3</td>
<td>Wesolowski-Louvel et al. (1992b)</td>
</tr>
<tr>
<td>MW109-8C</td>
<td>MATa lysA1-1</td>
<td>Wesolowski-Louvel et al. (1992b)</td>
</tr>
<tr>
<td>MW346/5</td>
<td>MATa Ade- his3 lac4-8 leu2 uraA1-1</td>
<td>This work</td>
</tr>
<tr>
<td>MWK23</td>
<td>Isogenic to MW346/5 except rag3-Δ3::LEU2</td>
<td>This work</td>
</tr>
<tr>
<td>RV1</td>
<td>Isogenic to MW346/5 except rag3-Δ3::LEU2 Klgr1-1</td>
<td>This work</td>
</tr>
<tr>
<td>LT-16B</td>
<td>MATa lysA1-1 uraA1-1 rag3-Δ3::LEU2</td>
<td>This work</td>
</tr>
<tr>
<td>MW270-7B</td>
<td>MATa leu2 metA1-1 uraA1-1</td>
<td>Billard et al. (1996)</td>
</tr>
<tr>
<td>FRVR-2C</td>
<td>MATa lac4-8 uraA1-1 RAG3 Klgr1-1</td>
<td>This work</td>
</tr>
<tr>
<td>FRVR-5A</td>
<td>MATa lac4-8 his3 RAG3 Klgr1-1</td>
<td>This work</td>
</tr>
<tr>
<td>MWK14</td>
<td>Isogenic to MW270-7B except rag3-Δ2::URA3</td>
<td>Tizzani et al. (1998)</td>
</tr>
<tr>
<td>HNK1</td>
<td>Isogenic to MW270-7B except Rag1::KanMX4</td>
<td>Neil et al. (2004)</td>
</tr>
<tr>
<td>HNK14</td>
<td>MATa uraA1-1 lysA1-1 Klgr1-1::KanMX4</td>
<td>This work</td>
</tr>
<tr>
<td>MW364-14C</td>
<td>MATa Klgr1::KanMX4 rag3-Δ2::URA3</td>
<td>This work</td>
</tr>
<tr>
<td>MW365-1A</td>
<td>MATa rag3-Δ3::LEU2 Klgr1-1 uraA1-1 his3 metA1-1</td>
<td>This work</td>
</tr>
<tr>
<td>MW386-2B</td>
<td>MATa Klgr1-1 uraA1-1 his3 lysA1-1 leu2</td>
<td>This work</td>
</tr>
<tr>
<td>MWK15</td>
<td>Isogenic to MW270-7B except sck1::LEU2</td>
<td>Lemaire et al. (2002)</td>
</tr>
</tbody>
</table>

*Previously MW270-7B/Δrag3.

†Previously MW270-7B/Δsck1.

\( a \) indicates the genetic locus of mating type `a` in yeast.

### Media

Rich medium (YP) was 1% Yeast Extract (Becton Dickinson) and 2% Peptone (Becton Dickinson) with glucose added to a final concentration of 2% (YPD); in some cases, the final glucose concentration was 5%. The Rag phenotype was tested on YP medium containing 5% glucose and 5 μM antimycin A (GAA medium). G418 medium was YPD supplemented with 200 μg mL\(^{-1}\) geneticin. Minimal media contained 0.67% Yeast Nitrogen Base (Becton Dickinson) and 2% glucose (SD medium), and were supplemented with auxotrophic requirements. Solid media contained 2% Bacto agar (Becton Dickinson). The sporulation and mating medium was 5% Malt Extract (Becton Dickinson) and 3% Bacto agar (Becton Dickinson). Synthetic medium lacking thiamine (–THI) was prepared as previously described (Sherman, 1991).

### Mutagenesis

Rag\(^{+}\) revertant clones were obtained by UV mutagenesis of the Rag\(^{+}\) MWK23 strain. Briefly, 2 mL of a stationary-phase culture in YPD was washed with sterile 0.4% NaCl,
resuspended in 20 mL of 0.4% NaCl, and subjected to UV treatment (254 nm, 0.007 J cm\(^{-2}\); Biolink Pharmacia) in a Petri dish. After irradiation, the suspension was transferred to a 50-mL tube and placed on ice in the dark for 2 h. Mortality of the irradiated cells (65%) was estimated by plating dilutions of treated and nontreated cells on YPD plates. About 10\(^5\) cells were plated on YPD plates and replica plated, after 2 days of incubation at 28 °C, on GAA and -THI plates. Rag\(^+\) / Thi\(^-\) revertant clones were selected and named 'RVR'.

**Mini-Mu transposition**

The lysogenic *E. coli* strain MC4100 was transformed with plasmid pLVF7. Mini-Mu transposition mutagenesis of pLVF7 was performed as previously described (Lemaire et al., 2002). The plasmids containing the transposon inserted in the suppressor gene were selected for their ability to restore the original recessive Rag\(^+\) phenotype in the RVR1 strain. These plasmids were directly sequenced from the ends of the transposon (Lemaire et al., 2002) in order to identify the suppressor gene.

**RNA preparation and Northern blot analysis**

For RNA extraction, cells were pregrown overnight to an OD\(_{600}\ nm\) of 0.5–1 in YP medium. Then, glucose was added to a final concentration of 5%, and the cultures were further incubated for 3 h before the cells were harvested. Total RNAs extracted using the hot phenol procedure (Köhrer & Domdey, 1991) were fractionated by agarose/formaldehyde gel electrophoresis and transferred onto nylon membranes (Nytran-N; Schleicher and Schuell, Dassel, Germany). DNA probes were obtained either by digestion of plasmids or by PCR amplification of the genetic loci from the chromosomal DNA preparation. DNA fragments were labeled using \(\gamma\)-\[^{32}\]P\]dCTP (3000 Ci mmol\(^{-1}\)) and the Random Primed DNA labeling kit (Roche, Mannheim, Germany). The resulting \[^{32}\]P-labeled probes were hybridized to blotted membranes. Hybridization signals were detected by autoradiography. The *KIPDC1* probe was a 1.6-kb fragment from plasmid pDCE4 (Table 2), encompassing 0.8 kb of the promoter and 0.8 kb of the coding region. The *KlACT1* probe was a 1.3-kb fragment from plasmid pAXII-14 (Table 2), containing intron I and exon II sequences (Deshler et al., 1989). The *RAG1*, *RAG5* KIENO probes were obtained by PCR amplification using oligonucleotides described in Lemaire & Wesolowski-Louvel (2004). Oligonucleotides used to amplify the *KlGPM* locus were 5\(’\)-GAATTGTGG-AAGGAAAAACACG-3\(’\) and 5\(’\)-CCAAAATATGCCTAGC-GATTG-3\(’\).

**Enzymatic activities**

Cells were grown overnight in YPD medium, washed, and broken with a treatment with glass beads. PDC activity was measured as previously described (Bianchi et al., 1996). \(\beta\)-galactosidase activity was measured according to the method of Rose & Botstein (1983). Before cell treatment for \(\beta\)-galactosidase activity, aliquots of the cultures were diluted with sterile water and plated onto YPD plates and SD plates selective for transformed cells, in order to determine the percentages of vector-bearing cells (c. 50% stability). These values were used to evaluate specific \(\beta\)-galactosidase activities.

**Electrophoretic mobility shift assay (EMSA)**

The recombinant MBP-KlGcr1-1 fusion protein was expressed and purified as previously reported (Neil et al., 2004). DNA probes (200–400 bp) were PCR amplified using promoter sequences of the *KIPDC1*, *RAG5* and KIENO genes as templates and primers as previously described (Neil et al., 2004). End labeling of the PCR fragments and binding reactions were performed according to Neil et al. (2004). The samples were loaded on a 4% nondenaturing polyacrylamide gel and run in 0.5 × Tris-borate EDTA buffer for 2 h. The gel was fixed in 10% acetic acid, and scanned using a Cyclone Phosphoimager (Packard).

**Results and discussion**

**Isolation of an extragenic suppressor of the rag3 null mutation**

The rag3 null mutant strain MWK23 (Table 1) harboring Rag\(^+\) and Thi\(^-\) phenotypes was subjected to mutagenesis

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**Table 2. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDCE4</td>
<td><em>KIPDC1</em> probe: 1.6-kb EcoRI fragment in pBluescript II KS+</td>
<td>Bianchi et al. (1996)</td>
</tr>
<tr>
<td>pAXII-14</td>
<td><em>ACT1</em> probe: 1.3-kb HindIII fragment in pBluescript II SK+</td>
<td>This work</td>
</tr>
<tr>
<td>pLVF7</td>
<td>KCP491 library clone with a 13.4-kb insert containing <em>KLGCR1</em></td>
<td>This work</td>
</tr>
<tr>
<td>pMD12 and derivatives</td>
<td><em>KIPDC1</em> promoter::LacZ fusions in KCP491 vector</td>
<td>Destruelle et al. (1999)</td>
</tr>
<tr>
<td>pH14</td>
<td>pMAL-cRI carrying <em>malE::KLGCR1</em> fusion</td>
<td>Neil et al. (2004)</td>
</tr>
<tr>
<td>pNH14-1</td>
<td>pMAL-cRI carrying <em>malE::KlGcr1-1</em> fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pT218</td>
<td>pTZ18 carrying the <em>rag2::LEU2</em> disruption cassette</td>
<td>This work</td>
</tr>
<tr>
<td>pGEMgcr1-1</td>
<td>PCR-amplified <em>KlGcr1-1</em> cloned into pGEM-T Easy</td>
<td>This work</td>
</tr>
</tbody>
</table>
Revertant strains were crossed to each other, the RAGpressor mutation affected a single gene. When these three phenotype in the meiotic progeny, revealing that the sup-
RVR2, and RVR7) showed a 2:2 segregation of the RAG mutations were recessive. Three revertants (called RVR1, individually mated with the LT-16B/C13
recessive step. This was further confirmed by measuring PDC
activity in the isogenic triad RAG3 (MW346/5 strain), Δrag3
(MWK23 strain) and RVR1. The results (MW346/5 strain, 2340 ± 220 mU mg⁻¹; MWK23 strain, 1170 ± 50 mU mg⁻¹; RVR1 strain, 2320 ± 200 mU mg⁻¹) indicated that, in agreement with the transcription data, PDC activity was identical
in the wild-type and in the RVR1 strains, whereas it was reduced to one-half in the deleted strain.

The suppressor gene is KLGCR1

In order to identify the suppressor gene, the Rag⁺ RVR1 cells were transformed with a K. lactis genomic library based on a URA3 centromeric vector and screened for Rag transfectants (see ‘Materials and methods’). Ura⁺ transformant clones were tested for their Rag phenotype by replica-plating on GAA medium (see ‘Materials and methods’). One Rag⁻ clone was obtained, out of 2475 Ura⁺ clones. It contained a plasmid (pLVF7) bearing a 13.4-kb DNA sequence. The nucleotide sequence of the extremities of the pLVF7 insert revealed that it was a genomic fragment from chromosome E (http://cbi.labri.fr/Genolevures/).

The DNA spanned from the KLGCR1 locus to the KLLA0E23419g ORF, including genes orthologous to S. cerevisiae YTA6, UBP16 and BEM1. To determine which of these genes was the suppressor, plasmid pLVF7 was randomly mutagenized using mini-Mu transposition (see ‘Materials and methods’) and reintroduced into RVR1 cells. Insertions of the mini-Mu transposon that inactivated the wild-type suppressor gene were identified by the restoration of the Rag⁺ phenotype of the transformants. Plasmid analysis of these transformants, as well as subcloning from the pLVF7 plasmid, allowed the identification of KLGCR1 as the suppressor gene. KLGCR1 codes for a transcriptional regulator of glycolytic and KIPDC1 genes, homologous to ScGCR1, which both directly interact with their target promoters (Baker, 1991; Neil et al., 2004). The identity between KLGCR1 and the suppressor gene was further confirmed through genetic analysis. The revertant strain RVR1 (Δrag3 Klgcr1⁻1) was crossed with strain MW364-14C (Δrag3 ΔKlgcr1::KanMX4). In the meiotic segregant population (Fig. 2a), the Rag⁺ phenotype was never associated with G418 resistance in 30 tetrads dissected. This shows the absence of recombination between the suppressor mutation and the KLGCR1 locus, indicating that the two loci are tightly linked.

The Klgcr1⁻1 mutant allele from RVR1 was amplified, cloned, and sequenced. A mutation generating a stop codon at position 732 in the DNA-binding domain of the protein was found (Fig. 2b).

The Klgcr1⁻1 mutation was isolated from the Δrag3 mutation, through genetic crossing, in order to examine its phenotypes in a RAG3 background. Klgcr1⁻1 RAG3 strains (FRVR-2C and FRVR-5A) were constructed by crossing the MW109-8C and MKW23 strains (Table 1). The growth phenotypes presented in Fig. 2c show that Klgcr1⁻1 cells
behave like wild-type cells on both YPD and GAA media. This is in contrast with the ΔKlgcr1 cells, which grow poorly on YPD medium and display a Rag−/C0 phenotype (Neil et al., 2004). This result indicates that the Klgcr1-1 mutation, despite its recessiveness, is not a loss-of-function mutation. Klgcr1-1 can suppress all the rag3 mutant alleles

As Klgcr1-1 was isolated starting with a rag3 null mutation devoid of any Rag3 protein, we tested whether it could also suppress rag3 point mutations. In particular, we tested rag3-1 (PM6-7A/VV23 strain) and rag3-3 (PM6-7A/VV43 strain) alleles that retain 418 and 626 amino acids (out of 765), respectively, in the mutant proteins. PM6-7A/VV23 (Rag−) and PM6-7A/VV43 (Rag−) strains were crossed with a Δrag3 Klgcr1-1 Rag+ strain (MW365-1A). In both crosses, 2:2 segregation of the Rag phenotype was observed in the tetrads produced from meiosis of diploids. This result demonstrated that the Klgcr1-1 mutation was able to suppress all the rag3 mutant alleles. This was further confirmed by PCR amplification of the RAG3 locus that could discriminate between the null mutation and point
We investigated the putative DNA-binding domain of its C-terminal region. The truncated KlGcr1-1 has a deletion of 36 amino acids in its DNA-binding ability, although it was reduced in comparison to the wild-type protein.

The reduced affinity of KlGcr1-1 for the target DNAs could account for the recessiveness of the Klger1-1 mutation, as the wild-type KlGcr1 protein would compete and bind to them with greater affinity.

**The truncated KlGcr1-1 can activate glycolytic gene expression**

The DNA-binding ability of KlGcr1 could be essential for Klgcr1-dependent transcription. Therefore, we used the FRVR-2C and FRVR-5A strains (Klger1-1 RAG3) to test the effect of the Klger1-1 mutation on glycolytic gene transcription as well as RAG1 (glucose permease), the expression of which is indirectly affected by Klgcr1 (Neil et al., 2004). The transcription of RAG5 (hexokinase), KIENO (enolase), Kigpm (phosphoglyceromutase) and RAG1 appeared to be unaffected, or even slightly improved in the case of KIENO and RAG1 (Fig. 4). This result, showing that KlGcr1-1 can stimulate transcription of glycolytic and RAG1 genes, is in agreement with the growth phenotype of the cells on YPD medium and their Rag+ phenotype (Fig. 2c). These findings (ability of KlGcr1-1 to activate transcription despite its reduced binding to DNA) can be related to what has been reported for the KlGCR1 homolog ScGCR1 in S. cerevisiae (Tornow et al., 1993). In that case, it has been found that the complete removal of the DNA-binding domain of ScGcr1 does not affect its function, suggesting that it can be recruited to the promoter region by the DNA-binding protein Rap1, providing a heteromer.

**The Klger1-1 mutation suppresses the sck1 mutation**

We tested whether the suppressor effect of Klger1-1 was specific to the rag3 mutation or whether it could be extended to other glycolytic regulator mutations. The regulator that we examined was the transcriptional regulator of glycolytic genes, Sck1 (Lemaire et al., 2002), the mutant of which leads to a Rag+ phenotype. A cross was performed between the Rag- MWK15 strain (sck1Δ::LEU2) and the Rag+ MW386-2B strain (Klger1-1). Meiotic analysis showed that the Rag phenotype segregated 2+ : 2-, 3+: 1-, and 4+: 0-. Moreover, Leu+ spores could be Rag+, indicating that the Klger1-1 mutation could suppress the sck1 mutation. Therefore, the suppressor mutation can bypass both the rag3 and sck1 mutations, suggesting that the KlGcr1-1 mutant protein would be more efficient than the wild-type one, despite its reduced affinity for DNA.

Nevertheless, the isolation of Klger1-1 as an extragenic suppressor of the rag3 mutation is somewhat surprising, as Rag3 does not control any of the glycolytic genes (our unpublished results), except for KIPDC1.
The KIPDC1 promoter is a potential target of Rag3

Our results confirm that Rag3 has a role in fermentation by regulating the transcription of KIPDC1, even though its mechanism of action is not known at the molecular level. In addition, the identification of a DNA-binding protein as a suppressor, and the presence of a putative DNA-binding domain in the Rag3 protein, suggest that it would interact with the promoter of KIPDC1. Therefore, we tried to determine whether some specific region of KIPDC1 could be the target of Rag3.

The effects of RAG3 deletion and Klgcr1-1 suppression on transcription from the entire and deleted forms of the KIPDC1 promoter were analyzed. This was done by measuring the β-galactosidase activity of the reporter gene LacZ fused downstream of the promoter sequences on the centromeric vector KCp491 (Destruelle et al., 1999). The promoter deletions tested and the β-galactosidase activities are shown in Fig. 5a and b, respectively. Changes of the activity profile in Δrag3 transformants, vs. the wild-type transformants, were evident for the fusion containing the whole promoter sequence (pMD12) and for the deleted promoter sequences pMD8 and pMD12RR. These results suggest that Rag3 could interact with both the distal region of the promoter, lacking in pMD10, and with the internal region of the promoter, lacking in pMD12RR, but not with the ERA-like sequences, because no effect resulted from the deletion of the latter sequences (pMD12R).

The activity profile was completely restored in Δrag3 Klgcr1-1 transformants, except for the extended internal deletion of pMD12RR, indicating that this region was essential for the suppression and that it probably contained the sequences functionally interacting with the mutant protein KlGcr1-1. Results were identical with Klgcr1-1 transformants, suggesting that the internal region deleted in pMD12RR is required for KlGcr1-1 activity also in the presence of Rag3. These findings show that Rag3 would interact with KIPDC1 promoter.

Acknowledgements

Francesca Salani had a contract with the Centre of Excellence in Molecular Biology and Medicine of the University of Rome ‘La Sapienza’. Helen Neil was a recipient of a Fellowship from the Ministère de l’Education, de l’Enseignement Supérieur et de la Recherche. We would like to thank Claire Bärtschi for technical assistance. This work was partially supported by the Ministry of University and Research of Italy and by the Pasteur Institute Cenci-Bolognetti foundation.
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